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COLLAGEN SYNTHESIS AND DEGRADATION IN ACUTELY DAMAGED MOUSE LUNG TISSUE FOLLOWING TREATMENT WITH PREDNISOLONE

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Abstract—Corticosteroids are widely used to treat patients with acute lung damage. Recent work has shown that the administration of 30 mg/kg prednisolone to mice, twice daily on days 1–5 after the induction of lung damage with butylated hydroxytoluene (BHT), results in the development of a more severe fibrotic lesion [J. P. Kehrer, A. J. P. Klein-Szanto, E. M. B. Sorensen, R. Pearlman and M. H. Rosner, *Am. Rev. resp. Dis.* **130**, 256 (1984)]. In the present study, the rate of collagen synthesis in lung tissue from BHT–saline-treated mice was greater than that in lung tissue from oil-treated controls at all days examined. During prednisolone treatment, the rate of pulmonary collagen synthesis was significantly less in tissue from BHT–prednisolone-treated mice compared to BHT–saline controls. Two days after steroid treatment was stopped (day 7 after BHT), there was a significant increase in the rate of collagen synthesis in lung tissue from BHT–prednisolone-treated mice compared to tissue from both BHT- and oil-treated controls. This increase reached a maximum on day 11 and persisted to day 14 after BHT. The rate of pulmonary non-collagen protein synthesis was inconsistently increased in response to treatments with BHT and/or prednisolone. There was, therefore, a relatively greater increase in the synthesis of collagen. The percentage of total protein synthesis committed to collagen increased from 2% in oil-treated controls to 5% on day 7 after BHT alone and reached a maximum of 7.1% on day 11 in lung tissue from BHT–prednisolone-treated mice. The percentage of newly synthesized collagen that was degraded in lung tissue from BHT–prednisolone-treated mice was significantly lower than BHT–saline on days 7 and 11, and lower than oil–prednisolone on day 14. These results show that collagen synthesis was decreased in BHT-damaged mouse lung tissue during short-term, high-dose steroid therapy. There was, however, an increase in collagen synthesis and a decrease in the degradation of newly synthesized collagen after steroid therapy was stopped. These changes in collagen metabolism may contribute to the steroid-induced enhancement of fibrosis seen in BHT-treated mice.

Acutely damaged lung tissue can either repair itself with the return of normal pulmonary architecture and function, or it may begin to accumulate excess collagen with the development of pulmonary fibrosis [1–3]. The factors which determine the ultimate outcome of lung injury and the accumulation of collagen are not clear. Corticosteroids are the most widely used agents to treat patients with acute alveolar injury, although their efficacy in such disorders has never been demonstrated [4].

The deposition of excess collagen during the development of pulmonary fibrosis may result from an increased rate of collagen synthesis [1] and a decreased degradation of newly synthesized collagen [5–7]. Corticosteroids are presumed to be beneficial in patients with lung damage because of their anti-inflammatory activity, ability to decrease alveolar edema [8], inhibitory effects on collagen synthesis in various *in vitro* and *in vivo* systems [5, 9, 10], and ability to inhibit collagen accumulation in some models of fibrosis [11]. Recent work has shown, however, that while corticosteroids can inhibit collagen synthesis in normal lung tissue [12], collagen synthesis can be increased by these drugs in damaged lung tissue [12] and some other *in vitro* systems [13, 14].

Both the rate of collagen synthesis and the percentage of total protein synthesis devoted to collagen are increased in butylated hydroxytoluene (BHT)-damaged lung tissue [15]. The administration of high doses of prednisolone to mice for several days immediately after the induction of lung damage with BHT enhances the development of fibrosis [16–18], suggesting such treatments can produce delayed effects on collagen metabolism. The effects of corticosteroid therapy on these parameters and on the degradation of newly synthesized collagen are not known.

The data presented here show that during prednisolone treatment there were only small changes in the percentage and rate of collagen synthesis, and no changes in the degradation of newly synthesized collagen in normal or BHT-damaged lung tissue. Two days after prednisolone treatments ceased, the rate of collagen synthesis and the percentage of total protein synthesis devoted to collagen were greatly elevated in BHT-damaged lung tissue. Furthermore, the degradation of newly synthesized collagen was decreased. These results suggest a mechanism by which high-dose, short-term corticosteroid therapy could potentiate the deposition of collagen in acutely damaged lung tissue.

MATERIALS AND METHODS

Materials. Male, BALB/c mice, 8–12 weeks of age, were bred and maintained in the Animal Resources Center at The University of Texas at Austin. BHT, proline, 4-hydroxyproline, prednisolone, sodium dodecyl sulfate (SDS) and ninhydrin were obtained from the Sigma Chemical Co., St. Louis, MO. Dulbecco's Modified Eagle's Medium was obtained from GIBCO, Grand Island, NY, and *p*-dimethylaminobenzaldehyde from MCB, Cincinnati, OH. L-[5-^{3}H]Proline (21 Ci/mmole) was obtained from Amersham, Arlington Heights, IL. All other chemicals and solvents used were of reagent or high performance liquid chromotography (HPLC) grade.

Methods. BHT was dissolved in corn oil and administered to mice intraperitoneally at a dose of 400 mg/kg. Previous work has shown this dose to produce severe lung damage in mice [15, 19]. Prednisolone, 30 mg/kg, was administered subcutaneously as a suspension in isotonic saline at 8:00 a.m. and 4:00 p.m. on days 1–5 after BHT. This treatment regimen results in severe fibrosis within 2 weeks [16]. BHT and prednisolone concentrations were adjusted so that all injection volumes were 0.1 ml/10 g body weight. Control mice received equal volumes of vehicle.

Acid-soluble and acid-insoluble hydroxyproline synthesis, indexes of the degradation of newly synthesized collagen [20] and net collagen synthesis [21], respectively, were measured *in vitro* in minced lung tissue using the modifications of the method of Bradley *et al.* [22] described previously [15]. Lungs from three to four mice were pooled for each experiment and minced into pieces approximately 2 mm^3. Portions (200 mg) of the minced tissue were incubated at 37° in 0.5 ml of Dulbecco's Modified Eagle's Medium containing 100 μg/ml ascorbate and 10 μCi [5-^{3}H]-proline under an atmosphere of 95% O_2:5% CO_2. Flasks were analyzed after 1, 2 and 3 hr for hydroxyproline synthesis.

Minced lung tissue was next washed with cold phosphate-buffered saline (pH 7.4) and homogenized in 3 ml H_2O with a Tekmar Tissumizer (Cincinnati, OH). Proteins were precipitated by the addition of 1 ml of 20% trichloroacetic acid (TCA). After centrifugation at 1000 *g* for 5 min, the supernatant fraction was filtered through glass wool and 0.1 ml was analyzed for total radioactivity and 1.0 ml for free proline [23]. The remainder of the acid-soluble fraction was lyophilized and then hydrolyzed for 18 hr at 107° in 1 ml of 6 N HCl.

The TCA-insoluble pellet was washed two times with 5 ml H_2O, lyophilized, weighed, and hydrolyzed for 18 hr at 107° in 2 ml of 6 N HCl. The hydrolysate was neutralized with NaOH, treated with decolorizing carbon, filtered, and brought to a final volume of 4 ml with water. A 0.1-ml aliquot of this solution was analyzed for total TCA-precipitable radioactivity and a 2.0-ml aliquot was used to measure the quantity of TCA-insoluble [^{3}H]hydroxyproline [24]. The percentage of total protein synthesis committed to the synthesis of acid-insoluble collagen was calculated from the total TCA-insoluble radioactivity and the amount of TCA-insoluble [^{3}H]-hydroxyproline, using the factor 2.06 to correct for the differential incorporation of proline into non-collagen protein and into the hydroxyproline of lung collagen [25]. These data were also used to calculate non-collagen protein synthesis [25].

The hydrolysate of the TCA-soluble fraction was treated with decolorizing carbon and neutralized by passage over a 20-ml column of AG 11A8 ion-retardation resin (Bio-Rad Laboratories, Richmond, CA) [26]. Fractions (2 ml) were collected and all containing radioactivity were pooled and lyophilized. The radiolabeled hydroxyproline and proline present in this sample were separated by HPLC using a solvent-generated ion exchange system [26]. The sample was dissolved in 0.5 ml of 0.01 M $NaPO_4$, pH 2.8, containing 0.3% SDS. A 200-μl aliquot was injected onto a 15 cm × 4.6 mm Ultrasphere-ODS column (Beckman Instruments, Berkeley, CA) and eluted with the same buffer at a flow rate of 1 ml/min. Aliquots (1 ml) of the column effluent were collected and analyzed by liquid scintillation counting.

The percentage of the total radioactivity present in the acid-soluble fraction, recovered after this HPLC procedure, was determined by comparing the total hydroxyproline and proline counts in the HPLC column effluent with the counts determined in the original sample. Recoveries were in the range of 60–70%. Losses of proline and hydroxyproline occur proportionally [27], and this percentage was used to correct the final counts. The corrected proline counts, along with the measured free proline content of the acid-soluble fraction, were used to calculate the specific activity of this amino acid in the original incubation mixture. The degradation of newly synthesized collagen was expressed as the percentage of the total amount of [^{3}H]hydroxyproline synthesized which was acid soluble.

The rates of collagen and non-collagen protein synthesis were calculated by linear regression analysis, as described by Last *et al.* [28], using the quantities of hydroxyproline and proline incorporated into the acid-insoluble fraction after 1, 2 and 3 hr (plus a 0 hr value of 0 pmoles/mg dry wt). The slopes of the regression lines (rates of synthesis) were compared using a test analogous to Student's *t*-test [29]. The percentage of total protein synthesis devoted to collagen and the percentage degradation of newly synthesized collagen were calculated after 1, 2 and 3 hr of incubation for each treatment group. These data were then analyzed at each treatment day by one-way analysis of variance, and multiple comparisons were done with the Student–Newman–Keuls test [30]. A P value of less than 0.05 was considered significant.

RESULTS

Compared to oil-treated control mice at each treatment day, there was a significant increase in the amount of free proline/mg dry wt, following the *in vitro* incubation, only in minced lung tissue obtained from mice 11 days after BHT-prednisolone (Table 1). Proline values were also greater, compared to BHT–saline, on day 11 in lung tissue from mice that received BHT–prednisolone. There were no

Table 1. Lung free proline content*

Day	Free proline (μg/mg dry wt) Treatment: Oil–Saline	Oil–Prednisolone	BHT–Saline	BHT–Prednisolone
3	0.28 ± 0.04	0.27 ± 0.01	0.32 ± 0.02	0.28 ± 0.01
7	0.33 ± 0.03	0.31 ± 0.03	0.44 ± 0.06	0.41 ± 0.02
11	0.35 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	0.56 ± 0.05†‡
14	0.40 ± 0.01	0.46 ± 0.04	0.42 ± 0.06	0.49 ± 0.02

* Data are expressed as mean ± S.E. N = 3 for all points.
† Significantly different from oil-treated mice ($P < 0.05$).
‡ Significantly different from BHT–saline-treated mice ($P < 0.05$).

significant differences in free proline levels at each day among the other treatment groups nor following 1, 2 or 3 hr of incubation.

Time-dependent changes in the rates of acid-insoluble hydroxyproline synthesis in lung tissue from mice treated with BHT and prednisolone are shown in Fig. 1. The rate of hydroxyproline synthesis was elevated significantly, compared to oil-treated controls, 3 days after BHT–saline and remained elevated to day 14. The rate of hydroxyproline synthesis in lung tissue from mice treated with BHT–prednisolone was not significantly different from oil-treated controls on day 3 and was significantly less than the rate measured in lungs from BHT–saline-treated mice at this time. On day 7 (2 days after the cessation of steroid treatment), the rate of hydroxyproline synthesis in lung tissue from mice treated with BHT–prednisolone was significantly greater than the rate in lung tissue from all other treatment groups. The rate of hydroxyproline synthesis reached a maximum on day 11 in BHT–prednisolone-treated mice and remained significantly greater than all other treatment groups to day 14. There were no significant differences between the two oil-treated control groups except on day 7 where oil–prednisolone was slightly, but significantly, less than oil–saline.

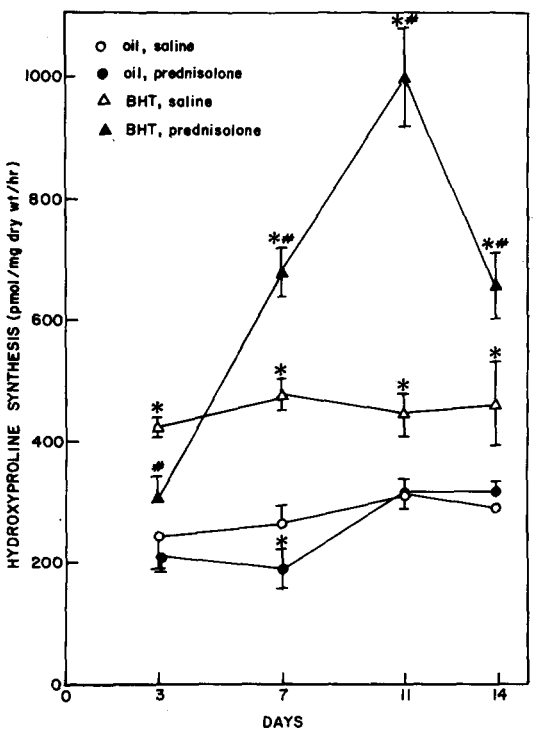

Fig. 1. Rates of pulmonary acid-insoluble hydroxyproline synthesis. Mice were injected i.p. with corn oil or 400 mg/kg BHT dissolved in corn oil. Prednisolone, 30 mg/kg, was injected s.c. twice daily on days 1–5. Data are expressed as slope ± S.E. N = 4 for all slope calculations except oil–saline, day 3, where N = 3. All correlation coefficients exceeded 0.973. Key: (*) significantly different from oil–saline-treated mice, and (#) significantly different from BHT–saline-treated mice ($P < 0.05$).

The rate of pulmonary non-collagen protein synthesis was elevated on days 3 and 11 after BHT–saline. Lung tissue from BHT–prednisolone-treated mice had a lower rate of non-collagen protein synthesis, compared to tissue from BHT–saline-treated mice, on days 3 and 14. Non-collagen protein synthesis following BHT–prednisolone was also less than oil-treated controls on day 14 (Table 2). There were no significant differences between the oil-treated controls at any day, although there was some day-to-day variability.

In contrast to these minimal effects on non-collagen protein synthesis, specific increase in collagen synthesis in lung tissue from BHT-treated mice could be seen when the synthesis of acid-insoluble [^{3}H]-hydroxyproline was expressed as the percentage of total protein synthesis devoted to the formation of acid-insoluble collagen (Fig. 2). Significant increases were seen on all days with BHT–prednisolone compared to oil-treated controls, and on days 11 and 14 compared to BHT–saline. A maximum of approximately 7.1% of total protein synthesis was committed to collagen on day 11 after BHT–prednisolone. The percentage of collagen synthesis after BHT–saline was increased, compared to oil-treated controls, only on days 3 and 7. There were no significant differences between the oil-treated controls except on day 3 when oil–prednisolone was slightly, but significantly greater than oil–saline. This statistical effect seemed to be due to the small variation among these particular samples and may have little biological significance.

There were no significant differences among the oil-treated control groups in the percentage of total [^{3}H]hydroxyproline synthesis which was acid-soluble, an index of the degradation of newly syn-

Table 2. Rates of non-collagen protein synthesis*

Day	Proline incorporated (nmoles/mg dry wt/hr) Treatment: Oil–Saline	Oil–Prednisolone	BHT–Saline	BHT–Prednisolone
3	7.9 ± 1.7	4.9 ± 0.7	11.7 ± 0.4†	9.2 ± 0.9‡
7	5.9 ± 0.5	4.9 ± 0.9	5.1 ± 0.2	5.6 ± 0.5
11	8.2 ± 0.1	9.1 ± 1.1	10.2 ± 0.5†	7.8 ± 1.5
14	9.3 ± 0.3	9.7 ± 0.6	9.7 ± 1.2	4.4 ± 0.9†‡

* Data are expressed as mean ± S.E.
† Significantly different from oil–saline ($P < 0.05$).
‡ Significantly different from BHT–saline ($P < 0.05$).

thesized collagen (Fig. 3). Collagen degradation in lung tissue from BHT–saline-treated mice was also not different from control, although a trend towards a decrease in the degradation of newly synthesized collagen was evident. There was significantly less degradation of newly synthesized collagen, compared to oil–saline, in lung tissue from BHT–prednisolone-treated mice on days 7 and 11, and compared to BHT–saline and oil–prednisolone on day 14.

DISCUSSION

Numerous models of pulmonary fibrosis have been shown to be associated with an increase in the rate of collagen synthesis [6, 15, 28, 31, 32]. Preventing this increase by the administration of corticosteroids should result in less collagen deposition. Corticosteroid therapy has been reported to inhibit the accumulation of collagen following the administration of bleomycin, and to either inhibit or have no effect on collagen synthesis [11, 33–35]. Methylprednisolone treatments have also prevented the increased rate of collagen synthesis in lung tissue from rats exposed to ozone [33]. These analyses were performed during the course of corticosteroid therapy, however, and little information is available on the rate of collagen synthesis in damaged lung tissue several days after steroid treatments cease.

Our recent report that short-term, high-dose prednisolone treatments can enhance the deposition of collagen in damaged lung tissue at later times [12] suggests that collagen synthesis rates may be altered after steroid therapy is stopped. Treatment of mice

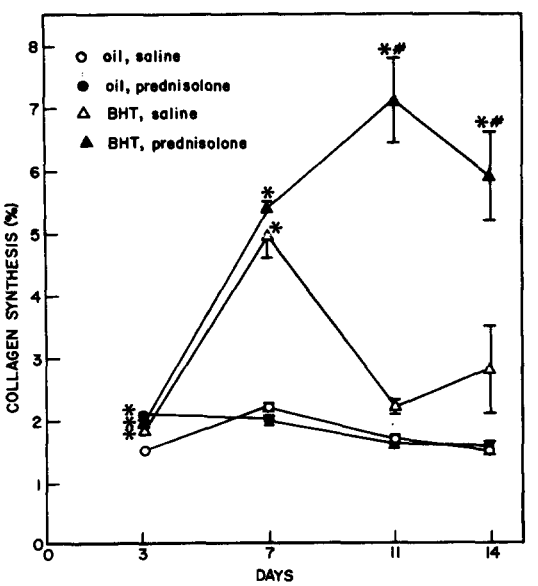

Fig. 2. Percentages of total protein synthesis committed to the production of acid-insoluble collagen in lung tissue. Mice were injected i.p. with corn oil or 400 mg/kg BHT dissolved in corn oil. Prednisolone, 30 mg/kg, was injected s.c. twice daily on days 1–5. Each point represents the mean ± S.E. of values obtained after 1, 2 and 3 hr of incubation. N = 3 for all points. Key: (*) significantly different from oil–saline-treated mice, and (#) significantly different from BHT–saline-treated mice ($P < 0.05$).

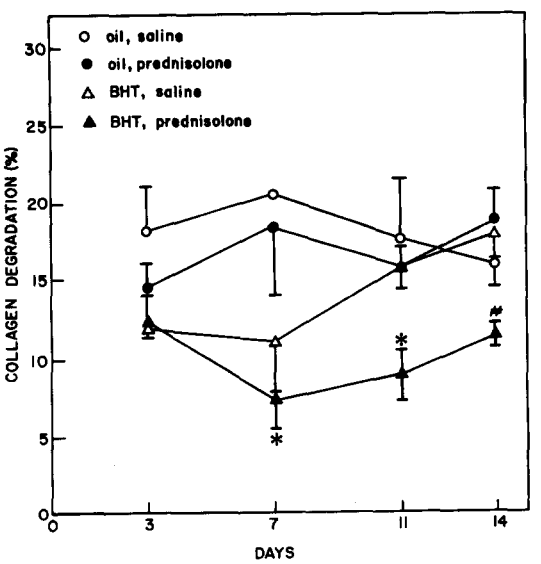

Fig. 3. Percentages of total [^{3}H]hydroxyproline synthesized which were acid-soluble. Mice were injected i.p. with corn oil or 400 mg/kg BHT dissolved in corn oil. Prednisolone, 30 mg/kg, was injected s.c. twice daily on days 1–5. Each point represents the mean ± S.E. of the values obtained after 1, 2 and 3 hr of incubation. N = 3 for all points except day 7, oil–saline, and days 11 and 14, oil–prednisolone, where N = 2. Key: (*) significantly different from oil–saline-treated mice, and (#) significantly different from BHT–saline- and oil–prednisolone-treated mice ($P < 0.05$).

with BHT–saline increased the rate of pulmonary collagen synthesis from days 3 to 14. Lung tissue from BHT–prednisolone-treated mice had a significantly slower rate of collagen synthesis on day 3 (during steroid therapy). This was followed, however, by a 2- to 4-fold increase in collagen synthesis after steroid therapy was stopped. These changes suggest that prednisolone will inhibit collagen synthesis while this drug is being given, but that increased and abnormal collagen synthesis begins once this inhibitory influence is removed.

Despite large increases in the rates of collagen synthesis, non-collagen protein synthesis showed inconsistent changes in response to the various treatments. Small changes in the incorporation of [^{3}H]-proline into total lung proteins greatly affects the calculation of non-collagen protein synthesis, however, and may, along with the small N values, explain this variability. Recent *in vivo* studies have shown that non-collagen protein synthesis is increased in BHT-damaged lung tissue (unpublished data), demonstrating that there is an increase in both collagen and non-collagen protein synthesis. There is, however, a relatively greater increase in collagen synthesis. The percentage of protein synthesis devoted to collagen ranged from 1.5 to 2.0% in control lung tissue and increased to 7.1% on day 11 in lung tissue from BHT–steroid-treated mice. The values for the control BALB/c mice used in these experiments were approximately twice those measured previously in CD-1 mice [15], but similar to the results in human [25] and rabbit [22] lung tissue.

Previous work has shown that BHT-damaged lung tissue has an increased amount of free proline both *in vivo* [7] and *in vitro* [12]. Similar results were seen in the current experiments although few increases were statistically significant, possibly due to the small N value. The increase in free proline in lung tissue from BHT–prednisolone-treated mice was greater than that in tissue from mice treated with BHT–saline. Although small, these increases occurred at the time of maximal collagen synthesis, supporting a contributory role [36].

Changes in the degradation of newly synthesized collagen, as well as changes in collagen synthesis, have been reported during the development of pulmonary fibrosis and may have a role in regulating the ultimate quantity of collagen that is deposited in damaged lung tissue [6, 7, 37]. Measurements of the degradation of newly synthesized collagen have used a variety of techniques [20, 38]. The data presented here were obtained using an HPLC procedure we have developed to accurately quantitate acid-soluble [^{3}H]hydroxyproline levels in the presence of large amounts of [^{3}H]proline [26, 27]. Using this method, the percentage of newly synthesized collagen that was degraded was close to the results reported by others [37], but significantly less than our previous work using a less precise procedure [7]. However, the same relative time-dependent changes were observed in the BHT–prednisolone model as in our earlier work on another model of pulmonary fibrosis.

The degradation of newly synthesized collagen was decreased significantly in lung tissue from mice given treatments that lead to severe fibrosis. This supports the work of Laurent and McAnulty who found a decreased rate of degradation in rats during the development of bleomycin-induced fibrosis [6], but contrasts with Clark *et al.* [39] who found an increased degradation of newly synthesized collagen in hamsters during the development of bleomycin-induced fibrosis. Explanations for this discrepancy may lie with differences in the species used, the model of fibrosis employed, or the method used to assess collagen degradation.

The present study shows that there was a large increase in the rate of pulmonary collagen synthesis in BHT-damaged mouse lung tissue shortly after prednisolone therapy was stopped. In addition, there was a decrease in the degradation of newly synthesized collagen in this tissue. Together, increased collagen synthesis and decreased degradation may be the proximate cause of the deposition of the excess collagen in these animals. It is also apparent that, while prednisolone therapy can appear to be beneficial while the therapy is in progress, measurements must also be made after treatments cease to observe potential delayed responses.

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